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REGULATION OF NEURAL STEM CELL PROLIFERATION

Reference to Related Applications

This is a continuation-in-part application of U.S. Ser. No. 08/270,412 filed July 5, 1994 which is a continuation application of U.S. Ser. No. 07/726,812 filed July 8, 1991. This application is also related to U.S. Ser. No. 07/951,813 filed October 16, 1992; U.S. Ser. No. 08/221,655 filed April 1, 1994; U.S. Ser. No. 08/010,829, filed January 29, 1993; U.S. Ser. No. 08/311,099, filed September 23, 1993; and U.S. Ser. No. 08/149,508, filed November 9, 1993.

Background of the Invention

In actively dividing tissues, such as bone marrow which gives rise to blood cells, specialized cells, known as stem cells, are present. The critical identifying feature of a stem cell is its ability to exhibit self-renewal or to generate more of itself. The simplest definition of a stem cell would be a cell with the capacity for self-maintenance. A more stringent (but still simplistic) definition of a stem cell is provided by Potten and Loeffler [*Development*, *110*: 1001 (1990)] who have defined stem cells as "undifferentiated cells capable of a) proliferation, b) self-maintenance, c) the production of a large number of differentiated functional progeny, d) regenerating the tissue after injury, and e) a flexibility in the use of these options."

Stem cells divide, generating progeny known as precursor cells. Precursor cells comprise new stem cells and progenitor cells. The new stem cells are capable of dividing again, producing more stem cells, ensuring self-maintenance, and more progenitor cells. The progenitor cells are capable of limited proliferation, where

all of their progeny ultimately undergo irreversible differentiation into amitotic, functional cells. FIG. 1 illustrates the relationship between stem cells, progenitor cells and differentiated cells.

The role of stem cells is to replace cells that are lost by natural cell death, injury or disease. The presence of stem cells in a particular type of tissue usually correlates with tissues that have a high turnover of cells. However, this correlation may not always hold as stem cells are thought to be present in tissues, such as the liver [Travis, *Science*, *259*: 1829 (1989)] that do not have a high turnover of cells.

The best characterized stem cell system is the hematopoietic stem cell. Evidence suggests that a single hematopoietic stem cell, located in bone marrow, is able to give rise, via a series of progenitor cells, to all of the blood cell lineages. US Patent 5,061,620, issued October 29, 1991, provides a means for isolating, regenerating and using the hematopoietic stem cell. Before birth, hematopoietic stem cells are active at many sites, including the fetal yolk sac, bone marrow, liver and spleen, Shortly before birth the bone marrow takes over as the primary site of hematopoiesis. The hematopoietic stem cells in the liver and spleen become quiescent and do not resume production of blood cells unless hematopoietic stem cell activity in the bone marrow is suppressed or widespread destruction of blood cells occurs.

The differentiated cells of the adult mammalian CNS exhibit little or no ability to enter the mitotic cycle and generate new neural tissue — essentially all neurogenesis occurs during the prenatal and immediate post-natal periods. While it is believed that there is a limited and slow turnover of astrocytes [Korr *et al.*, *J. Comp. Neurol.*, *150*: 169 (1971)] and that progenitor cells which can give rise to oligodendrocytes are present [Wolsqijk and Noble, *Development*, *105*: 386-698 (1989)], the generation of new neurons does not normally occur. Rats, however, exhibit a limited ability to generate new neurons in restricted adult brain regions such as the dentate gyrus and olfactory bulb [Kaplan, *J. Comp. Neurol.*, *195*: 323 (1987); Bayer, S.A, *NY. Acad. Sci.*, *457*: 163-172 (1985)] but this does not apply to all mammals; and the generation of new neurons in adult primates does not

occur [Rakic, P., *Science*, *227*: 1054 (1985)]. This inability to produce new neuronal cells in most mammals (and especially primates) may be advantageous for long-term memory retention; however, it is a distinct disadvantage when the need to replace lost neuronal cells arises due to injury or disease.

The low turnover of cells in the mammalian CNS together with the inability of the adult mammalian CNS to generate new cells in response to the loss of cells following injury or disease has lead to the assumption that the adult mammalian CNS does not contain stem cells. However, cells exhibiting stem cell characteristics *in vitro* have recently been isolated from the CNS. This cell is present in the embryo [Reynolds *et al.*, *J. Neurosci.*, *12*: 4565 (1992)] through to the adult [Reynolds and Weiss, *Science*, *255*: 1707 (1992)], suggesting that adult CNS, although it does not generate new cells in response to injury or disease, has the capacity to generate new cells and to repair itself via proliferation and differentiation of stem cells and their progeny in a manner analogous to the hematopoietic system. Recent results from *in vivo* experiments suggest that a population of relatively quiescent stem cells exist in the subependymal lining of the ventricles of the adult brain (Morshead *et al.*, Neuron, in press). It is possible that these stem cells, given the appropriate stimuli, could serve as a source of replacement cells in case of neural damage or disease.

Survival, expansion and proliferation of the hematopoietic stem cells, and stem cell systems in the liver, intestines and skin have been shown to be under the control of a number of different trophic factors. In the hematopoietic system, for example, growth factors such as erythropoietin and the glycoprotein CSF (colony-stimulating factor) and various interleukins have been identified as factors which regulate stem cell function [Metcalf, D., *Bioassays*, *14(12)*: 799-805 (1992)].

Research into the effects of trophic factors on neural cells during embryonic development suggest that endogenously occurring substances, such as platelet derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and nerve growth factor (NGF) participate in the prenatal

development of the nervous system. For example, a type of embryonic neural progenitor cell, known as the 0-2A cell, gives rise to oligodendrocytes and type-2 astrocytes. In the presence of PDGF, the 0-2A cell divides and after a few divisions differentiates into oligodendrocytes. The addition of CNTF and substrate factors, rather than PDGF, pushes the 0-2A progenitor cell to differentiate into type I astrocytes [Raff *et al.*, *Nature* (Lond.), *303*: 390-396 (1983)]. bFGF produces a two-told increase in the proliferation of embryonic progenitor cells which develop into neurons [Gensberger *et al. FEB Lett.*, *217*: 1-5 (1987)]. Cattaneo and McKay (1990) showed that growth factors added together or in sequential fashion will elicit novel responses not seen when the factors are added individually. They demonstrated that NGF stimulated the proliferation of embryonic neuroblasts to produce neurons only after they have been previously primed with bFGF [Cattaneo, E. and McKay, R., *Nature*, *347*: 762-765 (1990)]. bFGF has also been shown to influence the expression of the PDGF receptor and to block the differentiation of the 0-2A progenitor cell when exposed to PDGF [McKinnon *et al.*, *Neuron*, *5*: 603-614 (1990)]. EGF or TGFα show some mitogenic effects on embryonic retinal neuroepithelial cells grown in culture, resulting in progenitor cells which, in the continued presence of the growth factors, give rise to neurons but not to glial cells [Anchan *et al.*, *Neuron*, *6*: 923-936 (1991)]. In the same study, neurons and Müller cells are reported to occur in cultures derived from postnatal rat neuroepithelium.

CNS disorders encompass numerous afflictions such as neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), acute brain injury (e.g. stroke, head injury, cerebral palsy) and a large number of diseases associated with CNS dysfunction (e.g. depression, epilepsy, and schizophrenia). In recent years neurodegenerative disease has become an important concern due to the expanding elderly population which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease, Multiple Sclerosis, Huntington's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease, have been linked to the degeneration of neural cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended function. In addition to neurodegenerative diseases, acute brain injuries often result in the loss of neural

cells, the inappropriate functioning of the affected brain region, and subsequent behavior abnormalities. The most common types of CNS dysfunction (with respect to the number of affected people) are not characterized by a loss of neural cells but rather by an abnormal functioning of existing neural cells. This may be due to inappropriate firing of neurons, or the abnormal synthesis, release, and processing of neurotransmitters. Some of these dysfunctions are well studied and characterized disorders such as depression and epilepsy, others are less understood disorders such as neurosis and psychosis.

To date, treatment for CNS disorders has been primarily via the administration of pharmaceutical compounds. Unfortunately, this type of treatment has been fraught with many complications, including the limited ability to transport drugs across the blood-brain barrier and the drug-tolerance which is acquired by patients to whom these drugs are administered long-term. For instance, partial restoration of dopaminergic activity in Parkinson's patients has been achieved with levodopa, which is a dopamine precursor able to cross the blood-brain barrier. However, patients become tolerant to the effects of levodopa, and therefore, steadily increasing dosages are needed to maintain its effects. In addition, there are a number of side effects associated with levodopa such as increased and uncontrollable movement.

An emerging technology for treating neurological disorders entails the transplantation of cells into the CNS to replace or compensate for loss or abnormal functioning of the host's nerve cells. While embryonic CNS cells have given the best results in human trials [Winder *et al.*, *New Eng. J. Med.*, *327*: 1556 (1992)] and are the preferred donor tissue, ethical and political considerations, as well as the availability of sufficient quantities of tissue, limit the use of these cells. Other types of donor tissue for use in the treatment of CNS disorders are being developed. These include: genetically modified neural cell lines [Renfranz *et al.*, *Cell*, *66*: 173 (1991); Synder *et al.*, *Cell 68*: 1, (1992)], fibroblasts (Kawaja *et al.*, *J. Neurosci.*, *12*: 2849, (1992)], muscle cells [Jiao *et al.*, *Nature*, *363*: 456 (1993)], glial progenitor cells [Groves *et al.*, *Nature*, *362*: 453 (1993)] and encapsulated cells [Hoffman *et al.*, *Exp. Neurol.*, *132*: 100 (1993)].

While transplantation approaches represent a significant improvement over currently available treatments for neurological disorders, the technology has not yet been perfected. For example, upon transplantation, some cell types fail to integrate with host CNS tissue. In particular, the use of non-neuronal primary cell cultures limits the ability of the transplanted material to make connections with the host tissue. Immortalized donor cells obtained from primary neural tissue could form connections but the genetic expression of the oncogenes incorporated into these transformed cells is hard to control and could produce tumors and other complications. Donor and host could result in the rejection of the implanted cells. There is also the potential that the transplanted cells can result in tumor formation or pass infectious agents from the donor tissue to the host.

Gage *et al.*, in US Patent 5,082,670, disclose a method of treating defects, disease or CNS cell damage by grafting genetically modified neural cells into the appropriate CNS regions. The donor cells disclosed in this patent were obtained from non-neuronal primary cultures but it was suggested that genetically transformed neural cell lines could be used. These donor cell sources are inherently problematic. Gage *et al.* recognize the limitations imposed by the donor cells used in their technique and acknowledge that there is a ".. paucity of replicating non-transformed cell-culture systems..." They also recognize "the refractoriness of non-replicating neuronal cells to viral infection." This latter statement summarizes the difficulties associated with attempting to apply prior art methodology to genetically modify neural cells, which are not normally mitogenic unless obtained from embryonic tissue. Inherent in this technology is the potential for tissue rejection. Ideally, the genetically modified transplanted cells should be autologous, thereby preventing immunological complications — i.e. it would be beneficial if a patient's own quiescent neural stem cells could be genetically modified and/or stimulated to divide and differentiate, *in vitro*, into new neural cells which can then be implanted to replace lost or damaged neural tissue.

The multipotent neural stem cells, which are now known to be present in the brains of mammals throughout their lives [Reynolds and Weiss, *Science*, *255*: 1707 (1992)], provide a source of non-transformed neural cells which can be stimulated,

in the presence of a growth factor such as epidermal growth factor, to become mitotically active. In culture, the neural stem cells can be induced to proliferate and can provide large quantities of undifferentiated neural cells, which are capable of differentiation into the major types of neural cells and can be transplanted, genetically modified and then transplanted, or used for drug screening or other purposes.

It would be an advantage to be able to regulate the proliferation of neural stem cells *in vitro*, in order to be able to either increase, decrease or in some other way, alter, the mitotic activity of the neural stem cells and/or their progeny. Increasing the mitotic activity of quiescent neural stem cells would have an obvious benefit as the number of progeny available for transplantation, genetic modification, drug screening and so on, would be greater. It would also be advantageous to determine how proliferating neural stem cells, growing *in vitro* in the presence of a proliferation-inducing growth factor can be regulated to decrease the amount of proliferation. This information can be used to regulate, *in vivo*, the proliferation-inducing growth factors, such as those disclosed in U.S. Ser. No. 08/149,508 filed November 9, 1993. It would also be advantageous to be able to regulate not only the numbers of neural stem cells which become mitotically active in the presence of a growth factor or combination of growth factors, but to be able to regulate the rate of mitosis of the precursor progeny of these stems cells.

In view of the aforementioned deficiencies attendant with sources of CNS cells for transplantation or other uses, it should be apparent that a need exists in the art for reliable methods for culturing large quantities of embryonic and adult neural cells from human and non-human sources that have not been intentionally immortalized by the insertion of an oncogene in order to induce unlimited proliferation, thereby removing any question of the influence of genetic alteration on the normal function of the cells. There is also a need to be able to regulate the proliferation of the cells.

Accordingly, it is an object of this invention to provide a method for the *in vitro* regulation of the proliferation of CNS stem cells, by altering the culture medium in

which the stem cells are living through the addition of specific biological factors, such as growth factors or combinations of such factors.

This and other objects and features of the invention will be apparent to those skilled in the art from the following detailed description and appended claims.

None of the foregoing references is believed to disclose the present invention as claimed and is not presumed to be prior art. The references are offered for the purpose of background information.

Summary of the Invention

A method of regulating the *in vitro* proliferation of a multipotent neural stem cell and/or the proliferation of progeny of said neural stem cell is described. The method comprises the steps of dissociating mammalian neural tissue containing at least one multipotent neural stem cell capable of producing progeny that are capable of differentiating into neurons, astrocytes and oligodendrocytes, and proliferating the multipotent neural stem cell in a culture medium containing at least one proliferative factor that induces stem cell proliferation and a regulatory factor that regulates proliferation of the multipotent neural stem cell and/or proliferation of the progeny of the multipotent neural stem cell.

In one embodiment of the invention, the proliferative factor is bFGF and the regulatory factor is EGF or heparan sulfate which increase the rate of proliferation of stem cell progeny.

Brief Description of the Drawings

FIG. 1: A schematic diagram illustrating the proliferation of a multipotent neural stem cell. (A) In the presence of a proliferative factor the stem cell divides and gives rise to a sphere of undifferentiated cells composed of more stem cells and progenitor cells. (B) When the clonally derived sphere of undifferentiated cells is dissociated and plated as single cells, on a non-adhesive substrate and in the presence of a proliferative factor, each stem cell will generate a new sphere. (C)

If the spheres are cultured in conditions that allow differentiation, the progenitor cells differentiate into neurons, astrocytes and oligodendrocytes.

FIG. 2: (A) Photograph (100x magnification) of 10-day old neurospheres cultured in 20 ng/ml EGF. (B) Photograph (100x magnification) of 10-day old neurospheres cultured in 20 ng/ml FGF. (C) Photograph (100x magnification) of 10-day old neurospheres cultured in 20 ng/ml EGF + 20 ng/ml FGF.

FIG. 3: Graph showing the number of neurospheres generated from primary cells derived from the cervical, thoracic, and lumbar regions of adult mice spinal cord in the presence of 20 ng/ml EGF + 20 ng/ml FGF or 20 ng/ml FGF + 2 μg/ml heparan sulfate.

Detailed Description of the Invention

The invention is based on the development of procedures for regulating and manipulating the proliferation of multipotent neural stem cells and is directed towards regulating the numbers of progeny derived from a multipotent stem cell grown in culture. As used herein, the term "neural stem cell" or "central nervous system (CNS) stem cell" refers to a relatively quiescent, undifferentiated stem cell obtained from neural tissue that is capable of proliferation, giving rise to more neural stem cells (thus ensuring self-maintenance) and to progenitor cells. The term "multipotent" refers to a neural stem cell that is capable of producing progeny that give rise to each of the major types of differentiated neural cells, i.e. neurons, astrocytes and oligodendrocytes. In comparison, an undifferentiated cell that gives rise to two types of differentiated cells, for example, the O-2A cell, which gives rise to oligodendrocytes and astrocytes, is termed "bipotent", and one that gives rise to only one type of differentiated cell is termed "unipotent".

The term "progenitor cell" also refers to an undifferentiated cell derived from a neural stem cell but differs from a stem cell in that it has limited ability to proliferate and does not maintain itself. Each of a neural progenitor cell's progeny will, under appropriate conditions, differentiate into a neuron, astrocyte (type I or type II) or oligodendrocyte. Oligodendrocytes are differentiated glial cells that

form the myelin surrounding axons in the central nervous system (CNS). Oligodendrocytes have the phenotype galactocerebroside (+), myelin basic protein (+), and glial fibrillary acidic protein (-) [GalC(+), MBP(+), GFAP(-)]. Neurons are differentiated neuronal cells that have the phenotype neuron specific enolase (+), neurofilament (+), microtubule associated protein or Tau-1 (+) [NSE(+), NF (+), MAP-2 (+), or Tau-1 (+)]. Astrocytes are differentiated glial cells that have the phenotype GFAP(+), GalC(-), and MBP(-).

CNS stem cells have been reported and their uses described [Reynolds and Weiss, *Science*, *255*: 1707 (1992); Reynolds *et al.*, *J. Neurosci.*, *12*: 4565 (1992); Reynolds and Weiss, *Restorative Neurology and Neuroscience*, *4*: 208 (1992); Reynolds and Weiss, "Neuronal Cell Death and Repair" ed. Cuello, A.C., *Elsevier Science*, pp. 247-255 (1993)]. Additionally, the utility of these cells is described in published PCT applications no. WO 93/01275, WO 94/16718, WO 94/10292, and WO 94/09119. Like stem cells found in other mammalian tissues, the CNS stem cell is capable of self-maintenance and generating a large number of progeny including new stem cells and progenitor cells capable of differentiation into neurons, astrocytes and oligodendrocytes.

CNS stem cells can be isolated and cultured from any pre- or post-natal mammalian CNS tissue by the methods described by Reynolds and Weiss [*Science*, *255*: 1707, (1992)], the published PCT applications referenced above and in Example 1, below. Multipotent CNS stem cells occur in a variety of CNS regions, including the conus medullaris, cervical, thoracic and lumbar regions of the spinal cord, the brain stem, striatum and hypothalamus. The neural stem cells can be obtained from tissue from each of these regions and induced to divide *in vitro*, exhibiting self-maintenance and generating a large number of progeny which include neurons, astrocytes and oligodendrocytes.

In brief, the multipotent neural stem cell is obtained from neural tissue and grown in a culture medium which is preferably serum-free and which may comprise any combination of substances known to support the survival of cells. A suitable serum-free culture medium, herein after referred to as "Complete Medium",

comprises Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (Gibco) (1:1), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (5 mM) and a defined hormone mix and salt mixture (10%; available from Sigma), used to replace serum, which comprises insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 μM), putrescine (60 μM), and selenium chloride (30 nM). At least one biological factor that induces multipotent stem cell proliferation is added to the Complete Medium.

The term "biological factor", as used herein, refers to a biologically active substance that is functional in CNS cells, such as a protein, peptide, nucleic acid, growth factor, steroid or other molecule, natural or man-made, that has a growth, proliferative, differentiative, trophic, or regulatory effect (either singly or in combination with other biological factors) on stem cells or stem cell progeny. Examples of biological factors include growth factors such as acidic and basic fibroblast growth factors (aFGF, bFGF), epidermal growth factor (EGF) and EGF-like ligands, transforming growth factor alpha (TGFα), insulin-like growth factor (IGF-1), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and transforming growth factor betas (TGFβ); trophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF); regulators of intracellular pathways associated with growth factor activity such as phorbol 12-myristate 13-acetate, staurosporine, CGP-41251, tyrphostin, and the like; hormones such as activin and thyrotropin releasing hormone (TRH); various proteins and polypeptides such as interleukins, the Bcl-2 gene product, bone morphogenic protein (BMP-2), macrophage inflammatory proteins (MIP-1α, MIP-1ß and MIP-2); oligonucleotides such as antisense strands directed, for example, against transcripts for EGF receptors, FGF receptors, and the like; heparin-like molecules such as heparan sulfate; and a variety of other molecules that have an effect on neural stem cells or stem cell progeny including amphiregulin, retinoic acid, and tumor necrosis factor alpha (TNFα).

Biological factors, such as EGF and bFGF, that individually have a proliferative effect on multipotent neural stem cells are herein referred to as "proliferative

factors". Generally, proliferative factors bind to a cell-surface receptor, resulting in the induction of proliferation. Preferred proliferative factors include EGF, amphiregulin, aFGF, bFGF, TGFα, and combinations of these and other biological factors, such as heparan sulfate. A particularly preferred combination for inducing the proliferation of neural stem cells is EGF and bFGF. The proliferative factors are usually added to the culture medium at a concentration in the range of about 10 pg/ml to 500 ng/ml, preferably about 1 ng/ml to 100 ng/ml. The most preferred concentration for EGF, aFGF and bFGF is about 20 ng/ml of each proliferative factor.

The stem cells may be cultured in any culture vessels, for example 96 well plates or culture flasks. In the presence of a proliferation-inducing growth factor or combination of factors a multipotent neural stem cell divides, giving rise, within 3-4 days, to undifferentiated stem-cell progeny. The stem cell progeny, referred to herein as "precursor cells", include newly generated multipotent stem cells and progenitor cells. *In vitro*, the progeny of a single stem cell typically forms a cluster of precursor cells referred to herein as a "neurosphere"; however, culture conditions may be changed (e.g. by providing a treated substrate onto which the proliferating cells adhere) so that the proliferating cells do not form the characteristic neurospheres. Precursor cells are not immunoreactive for any of the neuronal or glial cell markers, but they are immunoreactive for nestin, an intermediate filament protein found in undifferentiated CNS cells [Lehndahl *et al.*, *Cell*, *60*: 585-595 (1990)].

In the continued presence of the proliferation-inducing growth factor, the precursor cells within the neurosphere continue to divide resulting in an increase in the size of the neurospheres as a result of an increase in the number of undifferentiated cells [nestin(+), NF(-), NSE (-), GFAP(-), MBP (-)]. It is possible to passage the precursor cells in the presence of the same growth factors or different growth factors that allow further proliferation to occur without promoting differentiation. Cells can be passaged 30 times or more using proliferative culture methods, resulting in an exponential increase in precursor cell numbers.

The culture techniques described above for the proliferation of CNS stem cells *in vitro* can be modified through the use of additional biological factors or combinations of factors which increase, decrease or modify in some other way the number and nature of the precursor cells obtained from the stem cells in response to EGF or other proliferative factors. Changes in proliferation are observed by an increase or decrease in the number of neurospheres that form and/or an increase or decrease in the size of the neurospheres (which is a reflection of the rate of proliferation — determined by the numbers of precursor cells per neurosphere). Thus, the term "regulatory factor" is used herein to refer to a biological factor that has a regulatory effect on the proliferation of stem cells and/or precursor cells. For example, a biological factor would be considered a "regulatory factor" if it increases or decreases the number of stem cells that proliferate *in vitro* in response to a proliferation-inducing growth factor (such as EGF). Alternatively, the number of stem cells that respond to proliferation-inducing factors may remain the same, but addition of the regulatory factor affects the rate at which the stem cell and stem cell progeny proliferate. A proliferative factor may act as a regulatory factor when used in combination with another proliferative factor. For example, the neurospheres that form in the presence of a combination of bFGF and EGF are significantly larger than the neurospheres that form in the presence of bFGF alone, indicating that the rate of proliferation of stem cells and stem cell progeny is higher.

Other examples of regulatory factors include heparan sulfate, transforming growth factor beta (TGFß), activin, bone morphogenic protein (BMP-2), ciliary neurotrophic factor (CNTF), retinoic acid, tumor necrosis factor alpha (TNFα), macrophage inflammatory proteins (MIP-1α, MIP-1ß and MIP-2), nerve growth factor (NGF), platelet derived growth factor (PDGF), interleukins, and the Bcl-2 gene product. Antisense molecules that bind to transcripts of proliferative factors and the transcripts for their receptors also regulate stem cell proliferation. Other factors having a regulatory effect on stem cell proliferation include those that interfere with the activation of the c-fos pathway (an intermediate early gene, known to be activated by EGF), including phorbol 12 myristate 13-acetate (PMA; Sigma), which up-regulates the c-fos pathway and staurosporine (Research

Biochemical International) and CGP-41251 (Ciba-Geigy), which down regulate c-fos expression and factors, such as tyrphostin [Fallon, D *et al.*, *Mol. Cell Biol.*, *11(5)*: 2697-2703 (1991)] and the like, which suppress tyrosine kinase activation induced by the binding of EGF to its receptor.

Preferred regulatory factors for increasing the rate at which neural stem cell progeny proliferate in response to FGF are heparan sulfate and EGF. Preferred regulatory factors for decreasing the number of stem cells that respond to proliferative factors are members of the TGFß family, interleukins, MIPs, PDGF, TNFα, retinoic acid (10^{-6} M) and CNTF. Preferred factors for decreasing the size of neurospheres generated by the proliferative factors are members of the TGFß family, retinoic acid (10^{-6} M) and CNTF.

The regulatory factors are added to the culture medium at a concentration in the range of about 10 pg/ml to 500 ng/ml, preferably about 1 ng/ml to 100 ng/ml. The most preferred concentration for regulatory factors is about 10 ng/ml. The regulatory factor retinoic acid is prepared from a 1 mM stock solution and used at a final concentration between about 0.01 μM and 100 μM, preferably between about 0.05 to 5 μM. Preferred for reducing the proliferative effects of EGF or bFGF on neurosphere generation is a concentration of about 1 μM of retinoic acid. Antisense strands, can be used at concentrations from about 1 to 25 μM. Preferred is a range of about 2 to about 7 μM. PMA and related molecules, used to increase proliferation, may be used at a concentration of about 1 μg/ml to 500 μg/ml, preferably at a concentration of about 10 μg/ml to 200 μg/ml. The glycosaminoglycan, heparan sulfate, is a ubiquitous component on the surface of mammalian cells known to affect a variety of cellular processes, and which binds to growth factor molecules such as FGF and amphiregulin, thereby promoting the binding of these molecules to their receptors on the surfaces of cells. It can be added to the culture medium in combination with other biological factors, at a concentration of about 1 ng/ml to 1 mg/ml; more preferred is a concentration of about 0.2 μg/ml to 20 μg/ml, most preferred is a concentration of about 2 μg/ml.

The precursor cells can be used for transplantation to treat various neurological disorders, as disclosed in PCT applications no. WO 93/01275, WO 94/16718, WO 94/10292, and WO 94/09119. The cells which are to be used for transplantation can be harvested from the culture medium and transplanted, using any means known in the art, to any animal with abnormal neurological or neurodegenerative symptoms, obtained in any manner, including those obtained as a result of chemical, electrical, mechanical or other lesions, as a result of experimental aspiration of neural areas or as a result of disease or aging processes.

The methods disclosed herein can also be used to test the proliferative or regulatory effects of biological factors on multipotent mammalian neural stem cell proliferation *in vitro*, prior to using the biological factors for the *in vivo* regulation of the proliferation of a patient's normally quiescent stem cells. The neural stem cells may be obtained from a human with a neurological disorder in order to test the proliferative or regulatory effects of biological factors on dysfunctional tissue.

Example 1

In vitro proliferation of multipotent CNS stem cells derived from embryonic brain tissue — neurosphere proliferation in response to EGF

Embryonic day 14 (E14) CD_1 albino mice (Charles River) were decapitated and the brain and striata removed using sterile procedure. The tissue was mechanically dissociated with a fire-polished Pasteur pipette into Complete Medium. The cells were centrifuged at 800 r.p.m. for 5 minutes, the supernatant aspirated, and the cells resuspended in Complete Medium for counting.

The cells were suspended at a density of 25,000 cells/ml in Complete Medium containing 20 ng/ml EGF. Using an Eppendorf repeat pipetter with a 5 ml tip, 200 μl of the cell suspension was added to each well of a 96 well plate with no substrate pre-treatment and housed in an incubator at 37°C, 100% humidity, 95% air/5% CO_2.

When the cells were proliferated, within the first 48 hours and by 3-4 days *in vitro* (DIV), they formed small clusters, known as neurospheres, that lifted off the substrate between 4-6 DIV. The number of neurospheres generated per well were counted and the results were tabulated and compared with the numbers of neurospheres generated in response to EGF after passaging the cells (see example 2) and in response to other biological factors alone, or in combination with EGF (see Example 3).

Example 2
Passaging of proliferated neurospheres

Paradigm 1: Cells and media were prepared as outlined in Example 1. Cells were plated at 0.2 x 10^6 cells/ml into 75 cm^2 tissue culture flasks (Corning) with no substrate pre-treatment and incubated as outlined in Example 1.

After 7 DIV, the neurospheres were removed, centrifuged at 400 r.p.m. for 2-5 minutes, and the pellet was mechanically dissociated into individual cells with a fire-polished glass Pasteur pipet in 2 mls of Complete Medium.

1 x 10^6 cells were replated into a 75 cm^2 tissue culture flask with 20 mls of the EGF-containing Complete Medium. The proliferation of the stem cells and formation of new neurospheres was reinitiated. This procedure can be repeated every 6-8 days.

Paradigm 2: The methods of Example 1 and Example 2 paradigm 1 were followed except that 20 ng/ml FGF was added to the Complete Medium in place of the EGF.

Paradigm 3: The methods of Example 1 and Example 2 paradigm 1 were followed except that 20 ng/ml FGF was added to the Complete Medium in addition to the 20 ng/ml EGF that was added.

Neurospheres, obtained after passaging, can be mechanically dissociated and the cells replated in 96 well plates as outlined in Example 1. The effects of specific

biological factors, or specific combinations of biological factors on the proliferation of neurospheres from cells derived from passaged neurospheres can be determined and compared with results obtained from cells derived from primary tissue.

Example 3

Assay of striatum-derived neurosphere proliferation in response to various combinations of proliferative and regulatory factors

Paradigm 1: Primary striatal cells prepared as outlined in Example 1 were suspended in Complete Medium, without growth factors, plated in 96 well plates (Nunclon) and incubated as described in Example 1. Following a one hour incubation period, a specific proliferative factor, or a combination of proliferative factors including EGF, or bFGF (recombinant human bFGF: R & D Systems), or a combination of EGF and bFGF, or EGF plus FGF plus heparan sulfate (Sigma), or bFGF plus heparan sulfate made up in Complete Medium at a concentration of 20 ng/ml for each of the growth factors and 2 μg/ml for heparan sulfate), was added to each well of the plate.

Activin, BMP-2, TGF-β, IL-2, IL-6, IL-8, MIP-1∂, MIP-1β, MIP-2 (all obtained from Chiron Corp.), TNFα, NGF (Sigma), PDGF (R&D Systems), EGF and CNTF (R. Dunn and P. Richardson, McGill University) were made up in separate flasks of compete medium to a final concentration of 0.2 μg/ml. Retinoic acid (Sigma) was added at a concentration of 10^{-6} M. 10 μl of one of these regulatory factor-containing solutions was added to each proliferative factor-containing well of the 96 well plates. Control wells, containing only proliferative factors, were also prepared.

In another set of experiments, the neurosphere inducing properties of each of these regulatory factors was tested by growing cells in their presence, in proliferative factor-free Complete Medium. None of these regulatory factors, with the exception of EGF, when used in the absence of a proliferation-inducing factor such as EGF or FGF, has an effect on neural stem cell proliferation.

The activin, BMP-2, TGF-β. IL-2, IL-6, IL-8, MIP-1∂, MIP-1β, MIP-2, TNFα and EGF additions were repeated every second day, CNTF which was added each day and retinoic acid, NGF and PDGF were added only once, at the beginning of the experiment. The cells were incubated for a period of 10-12 days. The number of neurospheres in each well was counted and the resulting counts tabulated using Cricket Graph III. Other relevant information regarding sphere size and shape were also noted.

In general, bFGF had a greater proliferative effect than EGF on the numbers of neurospheres generated per well. In the presence of 20 ng/ml EGF, approximately 29 neurospheres per well were generated. In the presence of bFGF, approximately 70 neurospheres were generated. However, in bFGF alone (FIG. 1B), the neurospheres were only about 20% of the size of those generated in the presence of EGF (FIG. 1A). The combination of EGF and bFGF (FIG. 1C) produces significantly more neurospheres than does EGF alone, but fewer than seen with bFGF alone. The neurospheres are larger than those seen in bFGF alone, approximating those seen in EGF. In the case of bFGF generated spheres, the addition of heparan sulfate increased the size of the spheres to about 70% of the size of those which occur in response to EGF. These data suggest that EGF and FGF have different actions with respect to the induction of stem cell mitogenesis.

The effects of the regulatory factors added to the proliferative factor-containing wells are summarized in Table I. In general, the TGFß family, interleukins, macrophage-inhibitory proteins, PDGF, TNFα, retinoic acid (10^{-6}M) and CNTF significantly reduced the numbers of neurospheres generated in all of the proliferative factors or combinations of proliferative factors tested. BMP-2 (at a dose of 10 ng/ml), completely abolished neurosphere proliferation in response to EGF. EGF and heparan sulfate both greatly increased the size of the neurospheres formed in response to bFGF (about 400%).

TABLE I

	PROLIFERATIVE FACTORS									
	EGF		bFGF		EGF + bFGF		bFGF + Heparan		EGF + bFGF + Heparan	
REGULATORY FACTORS	#	size	#	size	#	size	#	size	#	size
TGFβ Family ♦	-57%	-	-57%	-	-34%	--	-55%	-	-20%	-
BMP-2	-100%	n/a	-5%	=	+16%	--	-3%	-	+10%	--
Interleukins	-21%	=	-23%	=	-37%	-	-28%	=	-39%	-
MIP Family	-25%	=	-6%	=	-32%	-	-22%	=	-33%	-
NGF	-10%	=	0%	=	-30%	=	+5%	=	-48%	=
PDGF	-1.5%	=	-4%	=	-26%	=	-10%	=	-27%	=
TNFα	-17%	=	-17%	=	-41%	=	-21%	=	-37%	=
10^{-6}M Retinoic Acid	-8%	--	-61%	-	-31%	--	-65%	--	-45%	--
CNTF	-23%	-	-77%	~	-81%	--	-81%	-	-84%	--
EGF	-		-14%	+ +	-		-17%	=	-	
Heparan Sulfate	0%	=	0%	+ +	0%	=				

♦ Excluding BMP-2 (i.e. TGFα and activin)

Numbers of neurospheres generated (#) are given as percentages that reflect the decrease (-) or increase (+) in numbers of neurospheres per well, in response to a PROLIFERATIVE FACTOR in the presence of a REGULATORY FACTOR, compared with the number of neurospheres proliferated in the absence of the REGULATORY FACTOR.

Size of neurospheres generated in the presence of PROLIFERATIVE FACTORS and REGULATORY FACTORS compared to those generated in the presence of PROLIFERATIVE FACTORS alone are indicated as follows:

++: much larger; +: larger; =: approximately the same size; ~: variable in size; -: smaller; --: much smaller

Paradigm 2: Antisense/sense experiments: Embryonic tissue was prepared as outlined in Example 1 and plated into 96 well plates in Complete Medium. Antisense and sense experiments were carried out using the following oligodeoxynucleotides (all sequences written 5′ to 3′):

EGF receptor:	Sense strand:	GAGATGCGACCCTCAGGGAC
	Antisense strand:	GTCCCTGAGGGTCGCATCTC
EGF:	Sense strand:	TAAATAAAAGATGCCCTGG
	Antisense strand:	CCAGGGCATCTTTTATTTA

Each oligodeoxynucleotide was brought up and diluted in ddH_20 and kept at -20°C. Each well of the 96 well plates received 10μl of oligodeoxynucleotide to give a final concentration of either 1, 2, 3, 4, 5, 10 or 25μM. Oligodeoxynucleotides were added every 24 hours. The EGF receptor (EGFr) and EGF oligodeoxynucleotides were applied to cultures grown in bFGF (20 ng/ml), and EGFr oligodeoxynucleotides were applied to cultures grown in EGF (20 ng/ml). Cells were incubated at 37°C, in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well was counted and tabulated. A concentration of 3μM of antisense oligodeoxynucleotides produced a 50% reduction in the number of neurospheres generated per well, whereas the sense oligodeoxynucleotides had no effect on the number of neurospheres generated in response to EGF and FGF. Both the sense and antisense oligodeoxynucleotides were toxic to cells when 10μM or higher concentrations were used.

Similar experiments can be performed using the following oligonucleotides:

FGF receptor:	Sense strand:	GAACTGGGATGTGGGGCTGG
	Antisense strand:	CCAGCCCCACATCCCAGTTC
FGF:	Sense strand:	GCCAGCGGCATCACCTCG
	Antisense strand:	CGAGGTGATGCCGCTGGC

The FGF receptor (FGFr) and FGF oligodeoxynucleotides are applied to cultures grown in EGF, and FGFr oligodeoxynucleotides are applied to cultures grown in bFGF.

Paradigm 3: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. Complete Medium, containing 20 ng/ml of either EGF of bFGF is added to each well. 10μl of diluted phorbol 12-myristate 13 acetate (PMA) is added once, at the beginning of the experiment, to each well of the 96 well plates, using an Eppendorf repeat pipetter with a 500μl tip to give a final concentration of either 10, 20, 40, 100 or 200 μg/ml. Cells are incubated at 37°C in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days the number of neurospheres per well is counted and tabulated.

Paradigm 4: Embryonic tissue is prepared as outlined in Example 1 and plated into 96 well plates. 10μl of diluted staurosporine is added to each well of a 96 well plate, using an Eppendorf repeat pipetter with a 500μl tip to give a final concentration of either 10, 1, 0.1, or 0.001 μM of staurosporine. Cells are incubated at 37°C, in a 5% CO_2 100% humidity incubator. After a period of 10 to 12 days, the number of neurospheres per well is counted and tabulated.

Example 4
Adult spinal cord stem cell proliferation — *in vitro* responses to specific biological factors or combinations of factors

Spinal cord tissue was removed from 6 week to 6 month old mice, as follows: cervical tissue was removed from the vertebral column region rostral to the first rib; thoracic spinal tissue was obtained from the region caudal to the first rib and approximately 5 mm rostral to the last rib; lumbar-sacral tissue constituted the remainder of the spinal cord. The dissected tissue was washed in regular artificial cerebrospinal fluid (aCSF), chopped into small pieces and then placed into a spinner flask containing oxygenated aCSF with high Mg^{2+} and low Ca^{2+} and a trypsin/hyaluronidase and kynurenic acid enzyme mix to facilitate dissociation of the tissue. The tissue was oxygenated, stirred and heated at 30°C for 1 1/2 hours, then transferred to a vial for treatment with a trypsin inhibitor in media solution

(DMEM/12/hormone mix). The tissue was triturated 25-50 times with a fire narrow polished pipette. The dissociated cells were centrifuged at 400 r.p.m. for 5 minutes and then resuspended in fresh media solution. Cells were plated in 35 mm dishes (Costar) and allowed to settle. Most of the media was aspirated and fresh media was added. EGF alone, or EGF and bFGF were added to some of the dishes to give a final concentration of 20 ng/ml each, and bFGF (20 ng/ml) was added, together with 2 μg/ml of heparan sulfate, to the remainder of the dishes. The cells were incubated in 5% $C0_2$, 100% humidity, at 37°C for 10-14 days. The numbers of neurospheres generated per well were counted and the results tabulated. EGF alone resulted in the generation of no neurospheres from any of the spinal cord regions. In the presence of EGF plus bFGF, neurospheres were generated from all regions of the spinal cord, in particular the lumbar sacral region. The combinations of EGF + FGF and FGF + heparan sulfate produced similar numbers of spheres in the cervical region, whereas the combination of bFGF plus heparan sulfate resulted in fewer neurospheres from the thoracic and lumbar regions (see FIG. 3).

Example 5
Generation of neurospheres from primate tissue, *in vitro*, in response to proliferative factors

First-passage neurospheres were obtained from adult human tissue. During a routine biopsy, normal tissue was obtained from a 65 year old female patient. The biopsy site was the right frontal lobe, 6 mm from the tip of the frontal/anterior horn of the lateral ventricle. The tissue was prepared using substantially the same procedure outlined in Example 4 using aCSF. The stem cells were cultured in T25 flasks (Nunclon) in Complete Medium with 20 ng/ml EGF, 20 ng/ml bFGF, or 20 ng/ml each EGF plus bFGF. The flasks were examined every 2-3 days for neurosphere formation. More neurospheres were generated from the combination of EGF plus EGF than with either EGF or FGF alone.

All references cited herein are hereby incorporated by reference.